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# Fabrication and Comparison of Electro-Spun Poly Hydroxy Butyrate Valrate Nanofiber and Normal Film and its Cellular Study

**ALI MAJDI[1], ESMAEIL BIAZAR[2] and SAEED HEIDARI K.[3]**

[1]Young Researchers Club, Tonekabon Branch, Islamic Azad University, Tonekabon, (Iran).
[2]Department of Chemistry, Tonekabon Branch, Islamic Azad University, Tonekabon, (Iran).
[3]Proteomics Research Center, Faculty of Paramedical Sciences,
Shahid Beheshty University of Medical Sciences,Tehran (Iran).
E-mail e.biazar@tonekaboniau.ac.ir; Tel +98 1924271105; Fax +98 1924274409

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**ABSTRACT**

Tissue engineering is defined as the designing and engineering of structures to rebuild and repair a body damaged tissue. Scaffolding Poly Hydroxy Butyrate Valrate (PHBV) has shown good biocompatibility and biodegradable properties. Nanofibers have improved the performance of biomaterials, and could be considered effective. One of the important methods for designing nanofiber scaffold is the electrospinnig method. In this study , PHBV nanofibers were well designed. The samples were evaluated by SEM, contact angle and finally, cell culture. The SEM images showed the size average of nanofibers as to be about 280 nm. Contact angle analysis showed 67 degree for nanofibers and 86 degree for normal films. Cellular investigations (USS cells) showed better adhesion and cell growth and proliferation of nanofiber surfaces than normal samples.

**Key words:** PHBV, nanofiber, electrospinning, USSCs, cellular investigation.

## INTRODUCTION

Scaffold is one of the important factors in tissue engineering. Scaffolds must be have unique features in accordance with the damaged tissue[1]. Many methods can used for design such as phase separation, solvent casting, fiber bonding and etc. Eelectrospinning is novel method for design of nanostructures in tissue engineering .[2-4] Poly Hydroxy Butyrate Valerate (PHBV) is a biomaterial that is used in a variety of applications including surgical sutures, wound dressing, drug delivery and tissue engineering. This is due to its specific properties such as good biocompatibility, biodegradablity, non toxicity as well as its piezoelectricity features. However, this material is a hydrophobic polyester which should be modified with other materials until it improves its cell adhesion and hydrophilicity properties.[5-9] It has been generally accepted that extracellular matrix mimics may improve the attachment, proliferation, and the viability of the cultured cells.[10] Electro-spinning has

been rapidly developed into a technique to prepare nanofibers with the diameter ranging from tens of nanometers to several microns[11-13]. Therefore; during the last few years, many works considering the tissue engineering of electro-spun nanofibers have been reported. Most recently, electro-spun nanofibers were prepared by Yang et al. and were applied in neural tissue engineering. Although the presented nanofibers may mimic the morphologies of extracellular matrix to some extent, some modifications are still required to create a friendly environment for the cells' attachment, proliferation, and functions such as communications. [14,15] In this study, the USS cells were used. The Unrestricted somatic stem cells (USSCs) were first isolated from the umbilical cord blood in 2003 by Jager et al., and their differentiation capacity for transplantation was evaluated[16]. Koghler et al. (2004) also evaluated these cells for cytokine production. The USSCs are pluripotent, also, they are considered as the rare cell populations in umbilical cord blood. They have a high potential to proliferate and differentiate, thus, they are considered as valuable sources in cell therapy[17].The USSCs are $CD45^-$, the adherent and HLA class II- negative stem cells with a long telomerase. Moreover, these cells possess a unique cytokine profile and have a high percentage of productions associated with self-renewing factors. In spite of other cord blood-derived mesenchymal cells, differentiated only into osteoblasts, chondrocytes, adipocytes [18], and neurons [17] the USSCs have the differentiation potential into osteoblasts, chondrocytes, blood cells, neurons, hepatocytes, and heart tissues under *ex vivo* conditions[17]. This cell expresses different factors, including adherent cells, growth factors, and various cytokines such as SCF, VEGF, GM-CSF, M-CSF, TGF-1β, IL-6, G-CSF, LIF, Flt3 ligand, TPO, HGF, SDF-1α, IL-15, IL-12, IL-8, and IL-1β[18].

In this study, the PHBV nanofibers were obtained through the electrospinning method. The samples were evaluated by SEM and the contact angle also the cell culture with two USS cells.

## MATERIAL AND METHODS

### Nanofiber Preparation

A poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) PHBV containing 5 mol% of 3-hydroxyvalerate with 680,000 molecular weight was purchased from Sigma Chemical Co. 2, 2, 2-trifluoroethanol (TFE) to prepare PHBV solution was also purchased from Sigma-Aldrich Chemicals and was used as received without further purification. Electrospinning apparatus used in this study prepared from asia nano meghyas company (Iran). PHBV was dissolved at determined concentration in TFE. The PHBV solution (2%w) was contained in a glass syringe controlled by syringe pump. A positive high voltage source through a wire was applied at the tip of a syringe needle. In this situation a strong electric field ( 20 kv) is generated between PHBV solution and a collector. When the electric field reached a critical value with increasing voltage, mutual charge repulsion overcame the surface tension of the polymer solution and an electrically charged jet was ejected from the tip of a conical shape as the Taylor cone. Ultrafine fibers are formed by narrowing the ejected jet fluid as it undergoes increasing surface charge density due to evaporation of the solvent. An electro spun PHBV nanofibrous mat was carefully detached from the collector and dried in vacuum for 2 days at room temperature to remove solvent molecules completely. The nanofibers designed with determined parameters (Syringe Size : 17 mm ,Collector speed: 1000 rpm , Injected speed: 2 mL/min , Syringe tip distance to collector : 75 mm , Voltage : 20 kv, Temperature: 30 °C, Time: 7 h).

### Scanning electron microscopy

The surface characteristics of various modified and unmodified films were studied by scanning electron microscopy (SEM; Cambridge Stereo-scan, model S-360; Cambridge Instruments, Wetzlar, Germany) to analyze the changes in the surface morphology. The films were first coated with a gold layer (Joel fine coat, ion sputter for 2 hours) to provide surface conduction before their scanning.

### Contact angle analysis

The sample surfaces static contact angles were investigated by a contact-angle-measuring device (Krüss G10; Krüss, Matthews,NC) following the sessile drop method.

### Cellular analysis

### Culture and isolation of the USSC from fresh umbilical cord blood

After consent from mothers, their umbilical cords were obtained from the cord vein. Only 40%

of the cord blood samples contained USSCs. The mean age of donors was 28 years. After collecting the samples, the red blood cells were lysed using ammonium chloride (NH4CL) and the isolation procedure continued by Ficoll. Then the samples were rinsed twice with sterile phosphate buffered saline (pH 7.4). After centrifuging, the resultant cells were placed in Dulbecco's modified Eagle's medium(DMEM) low glucose, which had been enriched with 100 nmol dexamethasone, 10% fetal bovine serum (FBS), penicillin, and streptomycin. The first medium exchange process was done for 24 hours, then every 4 days. When 80% of the flask surface area was covered by the cells, the cells were passed by using 0.25% trypsin and ethylenediaminetetraacetic acid (EDTA). The USSCs were regularly expanded on the culture medium; and the 37°C temperature and 5% of $CO_2$ were required for the growth. The USSCs were first trypsinized and counted.

The tubes, containing 105–106 cells, were incubated on a rocker rotator for 10 to 6 hours, centrifuged at 1000 rpm for 6 minutes, and 3% human serum was added to cell deposition thereafter. The resultant mixture remained at room temperature for 30 minutes. The cells were again centrifuged as above and the PBS was added to the cell deposition. The cell mixture was passed through a nylon mesh, then 100 µL of cells was added to each tube with the following antibodies: anti-CD90, anti-CD105, anti-CD166, anti-CD45, anti-CD73, and anti-CD34. Next, they were kept at 4°C out of light for 45 minutes. After washing, the cells were fixed in 100 µL of 1% paraformaldehyde. Finally, the flow cytometric analysis was carried out. the karyotype analysis was performed on USSC. The first and last passages were chosen for karyotype analysis. The cells were first placed in an incubator with 0.1 µg/mL colcemid for 3 to 4 hours. Next, they were trypsinized, and 0.075 of M KCL solution was added. The cells were incubated with 5% CO2 at 37°C for 20 minutes. In the next stage, methanol and acetic acids in ratio of 1:3 were added for fixing the samples. Finally, the cells were scattered over the slide surface and the chromosomes were subjected to karyotype analysis.

**Cell culture study on the polymer surface**

The tissue culture polystyrene (TCPS) as control samples were well cleaned and sterilized by the autoclave method. Individual samples were placed in Petri dishes using a sterilized pincer; the USSC suspension was transferred to a flask (25 cm) containing of DMEM, 2mML-glutamine, penicillin [100 µL/mL], streptomycin [100 µL/mL]) and FBS 10%. The suspension was then placed in an incubator (5% $CO_2$, 37°C). The USSCs were proliferated in the flask and were washed using the PBS. Then the trypsin enzyme/EDTA was added to the flask (37°C); and the flask was incubated for 2 minutes. The culture media (FBS/DMEM) was added to the flask, and the cells were gently pipetted. The cell suspension was transferred to a falcon tube (15 mL) and centrifuged (1410 rpm) for 5 minutes. The solution was removed and the precipitation was transferred to a new flask (75 cm) for re-culturing. Pieces of cell culture (1 ×1 cm) from the petri dish (control) and the main sample were placed individually in one of the Petri dish wells by using a sterilized pincer. 100,000 cells/well were seeded into a 24-well culture plate removed by pipette and poured onto the control and the main samples. Then all samples were placed in Memmert incubator at 37°C for 48 hours and studied using a ceti microscope (Wolf Laboratories, UK). Cell proliferation was determined by the MTT assay for viable cell numbers.

The MTT tetrazolium compound was reduced by living cells in a colored formazan product that was soluble in the tissue culture medium. The quantity of formazan product was directly proportional to the number of viable cells in the culture. The assays were performed by adding 20 µL of MTT solution (Sigma-Aldrich) and 200µL of fresh medium to each well after aspirating the spent medium and incubating at 37°C for 4 hours with protection from light. Colorimetric measurement of formazan dye was performed at a wavelength of 570 nm using a Rayto micro-plate reader.

## RESULTS AND DISCUSSION

**SEM Investigations**

Fig. 1 shows the Electron Microscopy Images of the normal and the nanofibers in different magnifications (1a ; PHBV film – 1b ; PHBV nanofiber: 1000× – 1c; PHBV nanofiber: 10000× – 1d; PHBV nanofiber: 20000×). The smooth and

homology nanofibers have been clearly shown in the Figs. The size average was obtained for the nanofibers to be about 280 nm.

**Contact Angle Results**

Table 1 shows the contact angle obtained for the normal film and the nanofiber. The contact angles of 86° and 67° were obtained for the normal film and the nanofibers, respectively. The 19° difference in the contact angle, obtained for the normal film and the nanofibers, shows a better hydrophilicity of the nanofibers than the normal film.

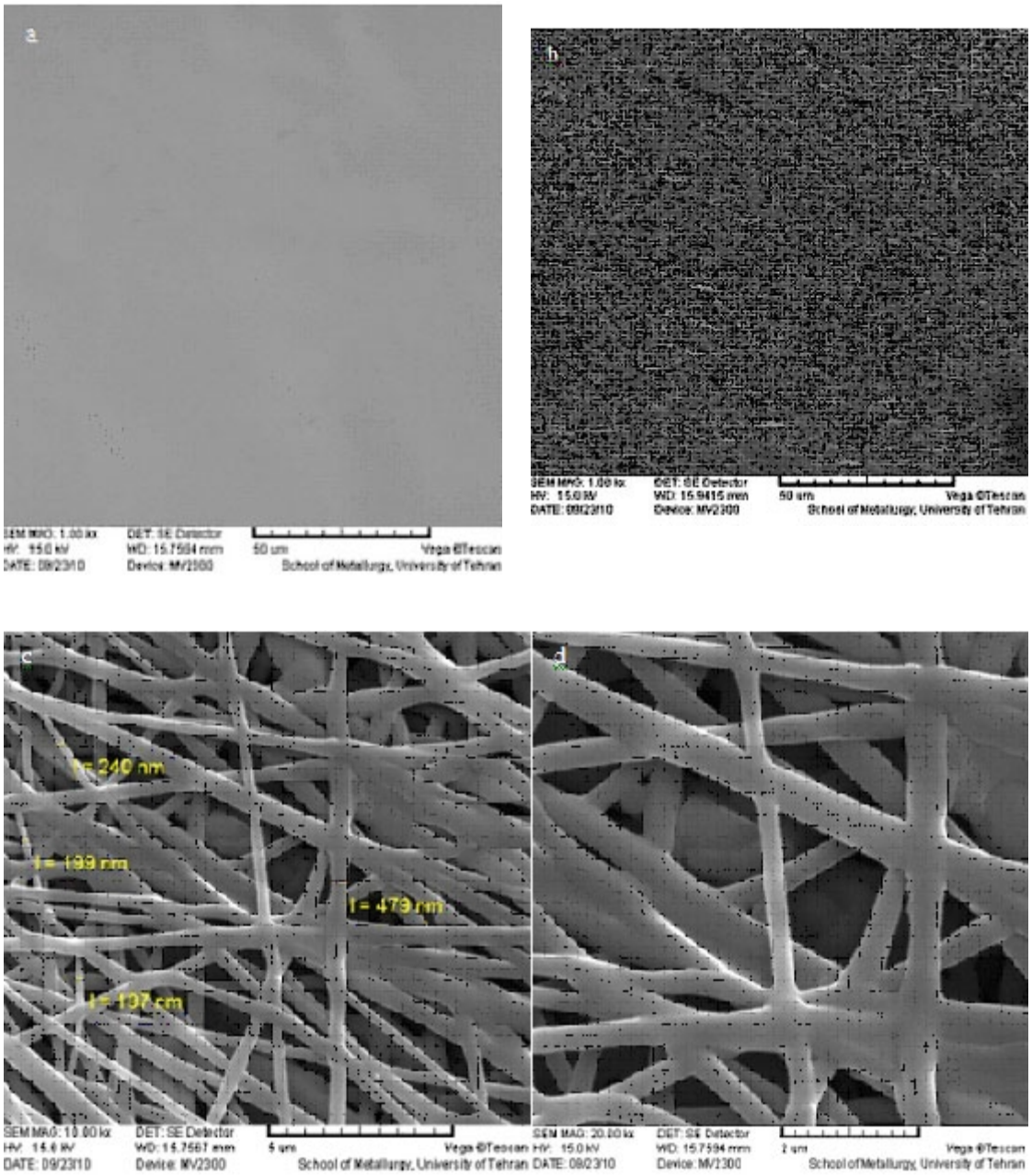


**Fig. 1: SEM images of PHBV normal film and nanofibers in different magnifications (1a ; PHBV film – 1b ; 1000× – 1c;10000× – 1d; 20000×)**

**Table 1 : Contact angle for PHBV normal film and nanofibers**

| T(°C) | PHBV normal film (degθ) | PHBV nanofiber (degθ) |
|---|---|---|
| 25 | 86 | 67 |

**Table 2: MTT analysis of the samples**

| Sampel | λ(nm) | Viability % |
|---|---|---|
| TCPS | 606 | 100 |
| PHBV film | 543 | 89 |
| PHBV nanofiber | 677 | 112 |

**Cellular Results**

The USSCs were isolated from the cord blood, and were then cultured. These cells were continuously cultured during 50 passages. The USSCs had a high proliferation capacity as they needed to repeat the passages. The proliferation and morphology of these cells were completely similar to each other before and after freezing. No indication of viral or mycoplasmal infection was observed during different stages of work on the USSC. The USSCs were morphologically adherent and spindle-shaped; they also had a size of 20-25 µm.

After 3 passages, the flow cytometric analysis was performed on the USSC in order to express the stem cell markers. The markers were as follows: CD34, CD45, CD73, CD105, CD90 and CD166. For the USSCs, the expression of the surface markers like CD90, CD105, CD166 and CD73 was positive, but was negative for CD34, CD45. Before beginning the experiments, the Karyotype analysis was performed on the USSCs of passage 2 which showed that they depicted a normal 44, XX Karyotype. After 48 passages, these cells were subjected to the Karyotype analysis once again, and it was indicated that they had a normal chromosome Karyotype of 44, XX.

Table 2 shows the MTT assay for TCPS (control), the normal film and the nanofiber samples. The results showed a high viability for the nanofibers samples than the normal films. Also, these nanofiber samples caused more cells to proliferate. Fig. 3 shows images of the cell culture on the normal film , the nanofibers and the control sample. Image A is related to the control sample and Image B is related to the PHBV normal film and the Images C is related to the nanofiber. Cellular images showed well growth in the vicinity of nanofibers.

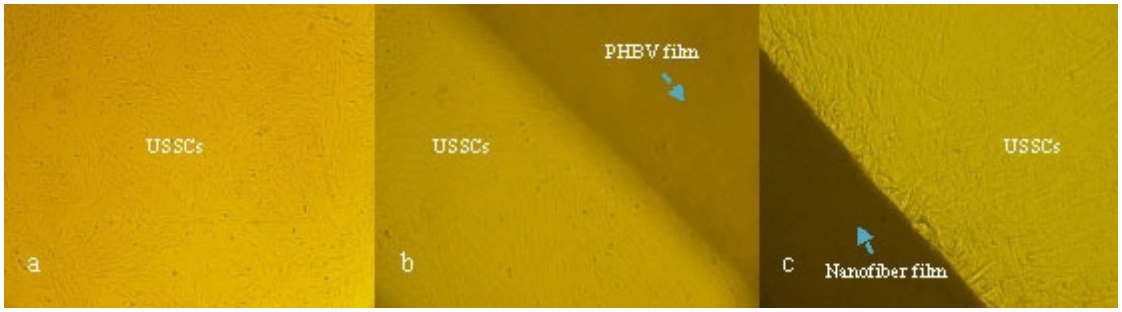


**Fig. 3: USS cell growth on the samples. Control a) TCPS b) PHBV normal film c) and PHBV nanofiber**

## CONCLUSION

In this study, the PHBV nanofibers with a size average about 280 nm were designed. The 19° difference in the contact angle, obtained for the normal film and the nanofibers, shows a better hydrophilicity of the nanofibers than the normal film. Cellular images showed well growth , adhesion and viability in the vicinity of nanofibers than the normal films. These PHBV nanofibers could be used well for tissue engineering.

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